Characterization of the cytotoxic effect of extracellular ATP in J774 mouse macrophages

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Extracellular ATP (ATP) is known to be cytotoxic to many cell types through a mechanism which is largely unknown. Very recently this nucleotide has been shown to cause cell death by apoptosis, probably by interacting with specific cell-surface receptors. In the present study we have investigated the mechanism of ATP-dependent cytotoxicity in the macrophage-like cell line J774. It has been previously reported that in this cell type ATP activates trans-membrane Ca2+ and Na+ fluxes and a drastic increase in the plasma-membrane permeability to hydrophilic solutes smaller than 900 Da. These changes are followed by cell swelling and lysis. We show in the present study that, although this nucleotide triggers a rise in the cytoplasmic Ca2+ concentration, neither cell swelling nor lysis is Ca2+-dependent. Furthermore, cell lysis is not dependent on Na+ influx, as it is not prevented by iso-osmotic replacement of extracellular Na+ with choline or N-methylglucamine. On the contrary, ATP-dependent cytotoxicity, but not the ATP+-dependent increase in plasma-membrane permeability, is completely abrogated in sucrose medium. Under our experimental conditions ATP does not cause DNA fragmentation in J774 cells. We conclude from these findings that ATP does not cause apoptosis of J774 macrophages and promotes a Ca2+- and Na+-independent colloidal-otic lysis.

INTRODUCTION

Several cell types are susceptible to the cytolytic activity of extracellular ATP (ATP+), but the mechanism whereby this nucleotide acts is poorly characterized (Steinberg & Di Virgilio, 1991). In J774 and other cell types the effects of ATP are mediated by specific cell-surface receptors (P2 purinergic receptors) coupled to different early responses: generation of inositol 1,4,5-trisphosphate, release of Ca2+ from intracellular stores, Ca2+ influx from the extracellular milieu, Na+ influx accompanied by plasma-membrane depolarization, and permeabilization of the plasma membrane to low-molecular-mass aqueous solutes (Gomperts, 1983; Steinberg & Silverstein, 1989; Dubyak, 1991). These changes could in principle be causally linked to cell death, as on the one hand it is thought that a perturbation of the plasma-membrane permeability leading to changes in intracellular ion homeostasis may cause colloidal-otic lysis, whereas on the other an increase in cytoplasmic free Ca2+ concentration ([Ca2+]i) and/or an inappropriate activation of protein kinase C have been invoked as triggers of apoptosis (McConkey et al., 1990). We have previously shown that ATP, promotes Ca2+-independent apoptosis of a number of tumoral cell lines (Zanovello et al., 1990; Murgia et al., 1992), and similar results have been reported in mouse lymphocytes and endotoxin-primed macrophages (Zheng et al., 1991; Hogquist et al., 1991). However, the present results show that ATP does not cause apoptosis of J774 mouse macrophages, as under no conditions were we able to detect the typical ladder-pattern of agarose-gel electrophoresis of DNA from cell extracts. Furthermore, ATP caused an early swelling, probably owing to opening of the ATP+-gated pore known to be expressed by these cells (Steinberg & Silverstein, 1987; Beyer & Steinberg, 1991), followed by disruption of intracellular organelles and the plasma membrane, changes indicative of colloidal-otic lysis. ATP+-triggered lysis was independent of the presence of extracellular Ca2+ and did not require a Na+ influx; furthermore, although ATP+ also caused a transient [Ca2+]i increase owing to Ca2+ release from intracellular stores, this fast [Ca2+]i transient was unlikely to be a trigger for cell lysis, since it also occurred in the ATP-resistant J774 variant ATPRB2 that was refractory to the cytotoxic effect of ATP+. Rather surprisingly, lysis of ATP+-pulsed cells was completely prevented in sucrose medium. Under these incubation conditions, the ATP+–pulsed pore was fully activated and the plasma membrane was permeabilized, as shown by uptake of extracellular markers and cell swelling, yet no release of lactate dehydrogenase occurred and cells remained viable for several hours. Our results suggest that the mechanism responsible for ATP+-dependent cell death is a feature of the target, and, very probably, depends on the P2 purinergic receptor subtype expressed on the plasma membrane.

MATERIALS AND METHODS

Cells

The J774 mouse macrophage cell line and the ATPRB2 J774 variant were grown in spinner cultures in Dulbecco’s modified Eagle medium supplemented with 10% (v/v) heat-inactivated horse serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Unless otherwise indicated, experiments were performed in a saline solution containing (in mmol/l): 125 NaCl, 5 KCl, 1 MgSO4, 1 Na2HPO4, 5.5 glucose, 5 NaHCO3, 1 CaCl2 and 20 Heps (pH 7.4, 37 °C). This saline medium is subsequently also referred to as standard saline. In some experiments NaCl was replaced by an iso-osmotic concentration of choline chloride, methylglucamine or sucrose. In these Na+-free buffers KCl was omitted, Na2HPO4 was replaced with K2HPO4 and NaHCO3 with KHCO3, and the pH was adjusted to 7.4 with Tris/HCl.

Enzyme release

Lactate dehydrogenase activity was measured by standard methods (Bergmeyer, 1963).

Abbreviations used: ATP, extracellular ATP; [Ca2+]i, cytoplasmic free Ca2+ concentration; Ca2+-, extracellular free Ca2+; Na+–, intracellular Na+; Na+, extracellular Na+.
Measurement of \([\text{Ca}^{2+}]_i\)

Loading with fura-2 acetoxymethyl ester and measurement of \([\text{Ca}^{2+}]_i\), were performed essentially as previously described (Di Virgilio et al., 1988). To prevent fura-2 leakage and sequestration, 250 \(\mu\text{M}\)-sulphinpyrazone was present throughout the loading procedure and \([\text{Ca}^{2+}]_i\), measurement (Di Virgilio et al., 1988). To measure \([\text{Ca}^{2+}]_i\), macrophages were suspended in a thermostatically controlled and magnetically stirred cuvette (Perkin-Elmer LS5) at a concentration of 0.5 \(\times\) 10^6 cells/ml.

Measurement of cell swelling

Cell volume changes were monitored by measuring right-angle light scattering, at a wavelength pair of 540/550 nm, in a thermostatically controlled and magnetically stirred fluorimeter (Perkin-Elmer LS5) cuvette at a concentration of 0.5 \(\times\) 10^6 cells/ml.

Measurement of Lucifer Yellow uptake

For Lucifer Yellow uptake, macrophage monolayers were incubated for 5 min at 37 °C in sucrose/saline in the presence of 5 mM-ATP_x, 1 mg of Lucifer Yellow/ml and 250 \(\mu\text{M}\)-sulphinpyrazone. After this incubation time, the monolayers were rinsed and kept in sulphinpyrazone-supplemented sucrose/saline.

Microscopy

Microscopic observations were performed with an Olympus (IMT-2 or BH-2) microscope equipped with a 40 \(\times\) objective.

Calculation of ATP_x

The ATP_x concentration, as a function of total ATP_x, \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) concentrations and pH, was determined as described by Fabiato (1988).

Data presentation

Results are expressed as means \(\pm\) S.D. of triplicate determinations from a single experiment representative of at least three similar.

RESULTS AND DISCUSSION

Fig. 1 shows a dose-response curve for ATP_x-dependent release of lactate dehydrogenase from J774 cells and the ATP-resistant variant ATPRB2 cells. After a 6 h incubation in the presence of this nucleotide (5 mM), about 50% of total cellular-lactate dehydrogenase content was released in the supernatant of J774 cells, but not ATPRB2 macrophages. Total ATP_x, threshold and EC50 for cytoxicity were about 750 \(\mu\text{M}\) and 1.5 \(\mu\text{M}\) respectively, corresponding to a concentration of ATP_x, the active ATP_x species, of 45 and 158 \(\mu\text{M}\) respectively, under our experimental conditions. As previously reported (Steinberg & Silverstein, 1989; Di Virgilio et al., 1989), other nucleotides, such as UTP, ITP and CTP, even at very high concentration, could not mimic the cytotoxic effect of ATP_x (results not shown). The cytotoxic effect of ATP_x was dependent on the presence of the membrane-permeabilizing P2z purinergic receptor, as the ATPRB2 mutant cell line, which was not killed by ATP_x, expresses a fully functional P2y receptor, but lacks the P2z (Greenberg et al., 1988).

We and others have shown that activation of ATP_x of the P2z receptor and the subsequent increase in plasma-membrane permeability occur rapidly and without lag (Buisman et al., 1988; Tatham & Lindau, 1990; Pizzo et al., 1991). Release of lactate dehydrogenase, on the contrary, started about 1 h after addition of a maximally stimulatory ATP_x concentration and increased steadily throughout the incubation (Fig. 2). Release of lactate dehydrogenase from ATP_x-treated ATPRB2 cells and from control J774 cells incubated in the absence of the nucleotide is shown for comparison.

It has been previously reported that ATP_x-mediated cytotoxicity in rat hepatocytes is dependent on the presence of extracellular \(\text{Ca}^{2+}\) (\(\text{Ca}^{2+}\_i\)) (Nagelkerke et al., 1989). In contrast with these data, Fig. 3 shows that macrophages were fully susceptible to lysis in the absence of \(\text{Ca}^{2+}\_i\), and if anything, release of lactate dehydrogenase was increased, probably because the ATP_x concentration was higher in the absence of \(\text{Ca}^{2+}\_i\). Occurrence of lysis in the absence of \(\text{Ca}^{2+}\_i\) does not rule out a trigger role for \(\text{Ca}^{2+}\_i\) in ATP_x-dependent lysis, as J774 cells possess ATP_x-releasable intracellular \(\text{Ca}^{2+}\) stores; however, in agreement with previous results of Greenberg et al. (1988), we also observed a transient ATP_x-stimulated (\(\text{Ca}^{2+}\_i\)), rise in the ATP_x-resistant ATPRB2 variant, thus suggesting that mobilization of intracellular \(\text{Ca}^{2+}\) is unlikely to be the trigger for lysis (results not shown). Furthermore, addition of the \(\text{Ca}^{2+}\) ionophore

Fig. 1. Dependency on the nucleotide concentration of lactate dehydrogenase release from J774 and ATPRB2 macrophages

Monolayers of 2 \(\times\) 10^6 cells/well were incubated in 24-well plates at 37 °C for 6 h in standard saline containing the indicated nucleotide concentration. At the end of the incubation, supernatants were collected, centrifuged to eliminate floating cells, and the lactate dehydrogenase (LDH) content was measured. LDH release is expressed as percentage of total content, determined by lysing an equal amount of cells with 0.1% Triton X-100. □, J774 cells; ■, ATPRB2 cells.

Fig. 2. Time course of lactate dehydrogenase (LDH) release from ATP_x-pulsed J774 and ATPRB2 macrophages

Macrophage monolayers (2 \(\times\) 10^6/well) were incubated as described in Fig. 1 in \(\text{Ca}^{2+}\_i\)-free 100 \(\mu\text{M}\)-EGTA-containing saline in the absence or presence of 3 mM-ATP_x. □, J774 cells; ■, ATPRB2 cells; ○, J774 cells incubated in the absence of ATP_x.
ionomycin after ATP, showed that both cell types possessed intracellular Ca2+ stores of comparable size.

ATP, has been shown to cause cell death by apoptosis of a number of cell types, such as lymphocytes, P-815 mastocytoma cells and L929 fibroblasts (Zanovello et al., 1990; Zheng et al., 1991; Murgia et al., 1992). However, this mechanism of cell death does not seem to operate in J774 macrophages, since we were unable to detect ATP,,-dependent DNA fragmentation under our experimental conditions (Fig. 4a). Furthermore, ATP, did not induce any of the morphological features of apoptosis, such as cell shrinkage and chromatin clumping; rather, it caused cell rounding, swelling and disruption of intracellular organelles (Fig. 4b), changes suggestive of a cytotoxic mechanism based on colloidio-osmotic lysis. ATP,-promoted volume changes started shortly after addition of the nucleotide, reached a plateau within 5 min and were partially reversed by addition of Mg2+ in excess of ATP, (Fig. 5, trace a). Cell swelling depended on the activation of the ATP,-gated pore (the P2 receptor), since ATPRB2 cells did not show any ATP,-triggered volume changes (Fig. 5, trace b).

Replacement of Na+, with other extracellular univalent cations did not abrogate ATP,,-mediated cytotoxicity, as release of lactate dehydrogenase was inhibited (by about 60%) in choline, but slightly enhanced (by about 25%) in N-methylglucamine medium (Fig. 6). On the contrary, iso-osmotic replacement with sucrose allowed a complete inhibition of cell death. A possible explanation for the protective action of sucrose could be that at low ionic strength ATP, was unable to activate the P2 receptor and thus trigger the plasma-membrane permeability changes involved in colloidio-osmotic lysis. However, this is not the case, as neither uptake of the extracellular fluorescent marker Lucifer Yellow nor cell swelling (Fig. 7) was inhibited in sucrose medium. As shown in Fig. 7, under these experimental conditions the increase in cell volume was not reversed by addition of Mg2+, probably because after resealing of the plasma membrane cells were unable to remove quickly the excess of osmotically active sucrose accumulated in the cytoplasm.

The cytotoxic activity of ATP, is long known, but little information is available on the plasma-membrane receptors, intracellular messengers and mechanism involved. To this purpose, the J774 mouse macrophage-like cell line is a very useful model, since it is equipped with well-characterized plasma-membrane ATP, receptors and fairly well-known trans-membrane signal-transduction pathways (Steinberg & Silverstein, 1989; Greenberg et al., 1988). Furthermore, Steinberg and
Silverstein have selected mutants which are completely resistant to ATP, and thus very helpful for investigating intracellular processes involved in ATP-dependent cytotoxicity (Steinberg & Silverstein, 1987; Greenberg et al., 1988). In J774 cells ATP does not cause any of the changes typical of apoptosis, such as cell shrinkage, chromatin condensation and DNA fragmentation. On the contrary, it promoted cell swelling, disruption of intracellular organelles and the plasma membrane, changes which are clear indication of colloidosmotic lysis. Rather intriguingly, Hogquist et al. (1991) reported ATP-triggered DNA fragmentation in mouse macrophages primed by a 48 h incubation in the presence of bacterial lipopolysaccharide, suggesting that activation by inflammatory agents can modulate macrophage responses to ATP. This conclusion is further supported by the observation that sensitivity to ATP is drastically enhanced in γ-interferon-primed human macrophages (Blanchard et al., 1991).

The intracellular processes triggered by ATP, and responsible for cell death are still unknown. A [Ca2+]i rise is clearly neither necessary nor sufficient, since on the one hand ATP is fully active in the absence of Ca2+, and on the other ATPRB2 mutants undergo ATP-stimulated [Ca2+] changes, yet they are not killed. Likewise, a Na+ influx is not required, as replacement with either choline or N-methylglucamine does not abrogate cytotoxicity. To our surprise, incubation in sucrose medium completely prevented lysis. Under these conditions the plasma membrane was fully permeabilized, as shown by cell swelling and uptake of extracellular markers, yet release of lactate dehydrogenase was abrogated. Incubation in sucrose medium may prove helpful to minimize cell damage during experiments involving protracted plasma-membrane permeabilization.

An open issue is whether the high ATP concentrations required to induce cell death are ever reached in vivo. ATP is known to be released from several cells, e.g. platelets, adrenal chromaffin cells, cytotoxic T lymphocytes and various nerve terminals (Dubayak, 1991). Basal ATP, plasma concentration is about 1–5 μM, whereas peaks up to 20 μM have been measured after extensive intravascular platelet aggregation (Born & Kratzer, 1984). However, it cannot be excluded that much higher ATP concentrations can be reached within protected environments, such as those formed during platelet–endothelial-cell or cytotoxic-target-cell interaction.
ATP-mediated lysis of macrophages

In conclusion, our results show that ATP, promotes Ca\(^{2+}\) and Na\(^{+}\)-independent cell lysis and strengthen the view that cytotoxic factors may use different cytocidal pathways in different cell targets.

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